Thymoquinone Nanoparticle Formulation and *In Vitro* Efficacy

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ABSTRACT

Thymoquinone (TQ) is a natural product with promising anticancer activity, but its development is hindered by its limited bioavailability. In this project, a nanoparticle formulation of TQ (TQ-NP) was formulated into nanoparticles using poly(styrene-b-ethylene oxide) (MW: 1500-2400 g/mole) with a TQ/polymer weight ratio of 1/1. The particle size determined by dynamic light scattering was 78 nm. The effects of TQ-NP versus free TQ were determined by MTT assay for MCF-7 and MDA-MB-231 cell lines. Treatment of both cell lines with 50-100 μM TQ-NP was significantly more potent in killing cells than treatment with the same concentrations of free TQ after 72 hours. These results suggest that a high TQ loading nanoparticle formulation provides enhanced antitumor activity *in vitro* when compared with free TQ.

Keywords: thymoquinone, nanoparticle, mcf-7, mda-mb-231

1 INTRODUCTION

Thymoquinone (TQ) is a plant derived natural product extracted from black seed essential oil [1]. Several studies have investigated its anticancer activity both *in vitro* and *in vivo* [2]. However, further development of thymoquinone is hindered by its limited bioavailability and low water solubility. Nanoparticle-based vehicles have been used to enhance the drug bioavailability and target the delivery of anti cancer agents selectively to tumor sites. These nanocarriers overcome the solubility issue commonly associated with cancer drugs and offer enhanced permeability and retention *in vivo* (EPR effect), resulting in preferential accumulation in tumor tissues compared with normal tissues [3]. Cancer drug nanoparticles can be formulated using lipid, biocompatible polymers, or other drug delivery platforms [3]. In polymer-based nanoparticles, a block copolymer with one hydrophobic and one hydrophilic block is used in an aqueous environment to maintain the drug in the core of the particle, with the hydrophilic block stabilizing the nanoparticle at the surface [4].

One method for the formation of polymer-based nanoparticles is Flash NanoPrecipitation (FNP). FNP is an easily scalable technique used for the generation of active solute nanocarriers for *in vivo* applications. In this technique, a water miscible solvent is used to dissolve the drug and a stabilizing amphiphilic molecule, such as a diblock copolymer with one hydrophilic and one hydrophobic block. The obtained solution is mixed at high intensity in a confined volume, providing a condition of high supersaturation, leading to the nucleation and growth of particles stabilized at the surface with the amphiphilic polymer. The result is high loading, high yield, controlled size nanoparticles [5].

The goals of this project are to develop a nanoparticle formulation of TQ (TQ-NP) with high TQ loading content using FNP, and to assess its *in vitro* activity against MCF-7 and MDA-MB-231 breast cancer cell lines.

2 MATERIALS AND METHODS

2.1 Preparation of Nanoparticles

Thymoquinone nanoparticles (TQ-NP) were prepared by FNP using a confined volume tangential flow mixing cell and controlled flow rates. The formulation was composed of TQ (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 50 mg/ml and the amphiphilic diblock polymer poly(styrene-b-ethylene oxide) (PS-PEO, Polymer Source, Dorval, Canada), with a polystyrene molecular weight of 1500 g/mole and polyethylene oxide molecular weight of 2400 g/mole) at a concentration of 50mg/ml using tetrahydrofuran (THF) as a solvent. The solvent stream was mixed with reverse osmosis grade water (18 mΩ) in a four-port mixer with flow rates of 12 ml/min for the THF stream and 108 ml/min for the water stream. Flow rates were controlled using two Harvard apparatus PHD2000 syringe pumps. The nanoparticle suspension was collected at the mixer outlet and stored in polypropylene tubes for analysis and testing.

2.2 Nanoparticles Size Measurement

Dynamic light scattering (DLS) was used to determine the hydrodynamic diameter of the nanoparticles. Measurements were performed at 25°C using a 90Plus/BI-MAS (BrookHaven Instruments Corporation (BIC), Holtsville, NY) and analyzed using the BIC particle sizing

software in automatic mode. The intensity of scattered light was detected at 90° to an incident beam. TQ-NP collected from the mixer outlet were diluted in water (1:10) prior to analysis. Size measurements are reported as average values of three runs.

2.3 Quantification of TQ

The amount of TQ was determined using High Performance Liquid Chromatography (HPLC, Agilent Technologies, 1100 series instrument, Walborn, Germany). TQ-loaded PS-PEO nanoparticles or free TQ stock were dissolved in THF (100%) to ensure complete TQ solubilization (dilution 1:10). The samples were then filtered through 0.2 µm syringe filter and injected into a BDS HYPERSIL C_{18} HPLC column (250 x 4.6 mm I.D, with 5 µm packing material purchased from Thermo Fisher Scientific, Germany) and eluted at 25°C using an isocratic mobile phase of water (> 18 MΩ resistivity): ACN (45: 55% v/v) at a flow rate of 1 ml/min. The injection volume was 20 µl and analysis was performed at 254 nm wavelength with a total run time of 12 min. Data acquisition, data handling and instrument control were performed using the Chemstation software package (Agilent).

TQ concentration in the formulation and the stock was calculated using a TQ calibration curve equation obtained by plotting the peak area of the analytical standards at 1.5, 3, 30, 75, 150, 300 and 500 µg/ml of TQ prepared in HPLC-grade acetonitrile against their TQ concentration. The response was linear in the range tested ($r^2 = 0.99927$).

2.4 Viability assay

The effects of TQ-NP versus free TQ were determined by MTT assay. Briefly, MCF-7 and MDA-MB-231 cells were seeded in 96 well plates at a density of 50,000 cells/ml. The cells were treated with TQ or TQ-NP at concentrations of 0, 10, 25, 50, 75 and 100 µM, respectively. At 24, 48 and 72 h post-treatment, the medium was removed and the cells were incubated with MTT solution (1 mg/ml in PBS) overnight. The viable cells were measured by their ability to reduce the yellow dye, MTT, to a purple formazan product. The MTT solution was therefore replaced by isopropanol to dissolve the formazan crystal formed. Finally, the formazan production was assessed by measuring the colorimetric absorbance of the different wells at 595 nm using a microplate reader. Cellular viability was expressed as a percentage of cell viability of TQ-treated cells relative to untreated controls. Experiments were carried in triplicate.

3 RESULTS

3.1 Nanoparticle Characterization

TQ-NP formed using TQ and PS-PEO via FNP resulted in a TQ/polymer weight ratio of 1/1, which is higher than typical TQ loading contents of ~1/10 reported in the literature [6]. DLS analysis revealed a nanoparticle hydrodynamic diameter of 78 nm and a PDI of 0.23. Particle size remained unchanged for at least two weeks with the sample stored at room temperature.

3.2 *In Vitro Cell Viability*

MCF-7 and MDA-MB-231 cell viability was evaluated following exposure to TQ-NP or TQ. The effect of PS-PEO nanoparticles formed using FNP (without TQ) on both cell lines was also tested and revealed no effect on viability relative to the control. The time- and dose-dependent effects observed for treatment with TQ and TQ-NP for the MCF-7 cell line are shown in Figure 1.

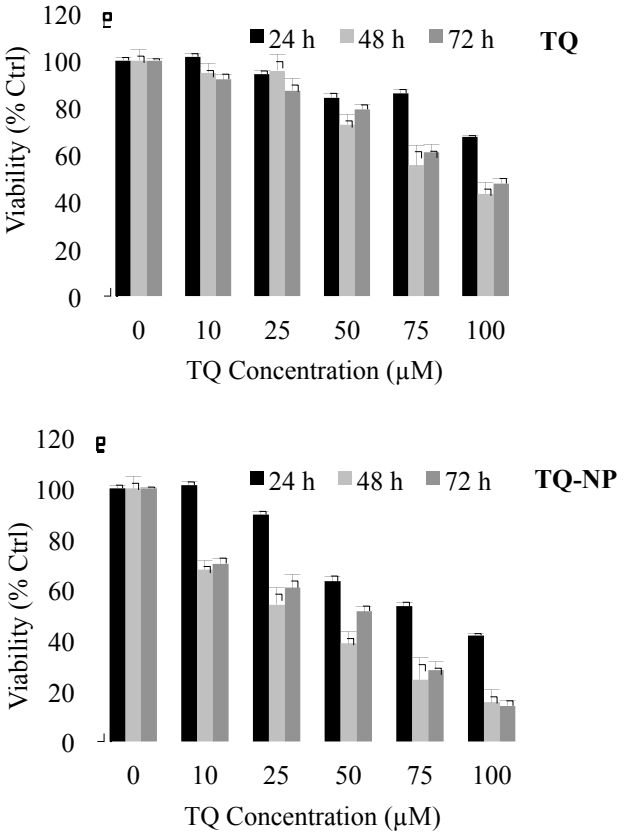

Figure 1: Effect of increasing TQ concentrations in TQ (top chart) and TQ-NP (bottom chart) formulations on the viability of MCF-7 cells as determined by MTT assay at 24, 48 and 72 h post-treatment.

For the MCF-7 cell line treated with TQ (Figure 1, top), the time effect is pronounced at TQ concentrations of 75

μM and 100 μM for the 24 h and 48 h time points, with no variation observed between the 48 h and 72 h time points. The dose dependence on cell viability is noticed at the 48 h and 72 h time points for concentrations of 50 μM and above.

For the MCF-7 cell line treated with TQ-NP (Figure 1, bottom), the time effect is pronounced at all TQ concentrations tested (10 μM to 100 μM) for the 24 h and 48 h time points, with no significant overall variation observed between the 48 h and 72 h time points.

The time- and dose-dependent effects observed for treatment with TQ and TQ-NP for the MDA-MB-231 cell line are shown in Figure 2.

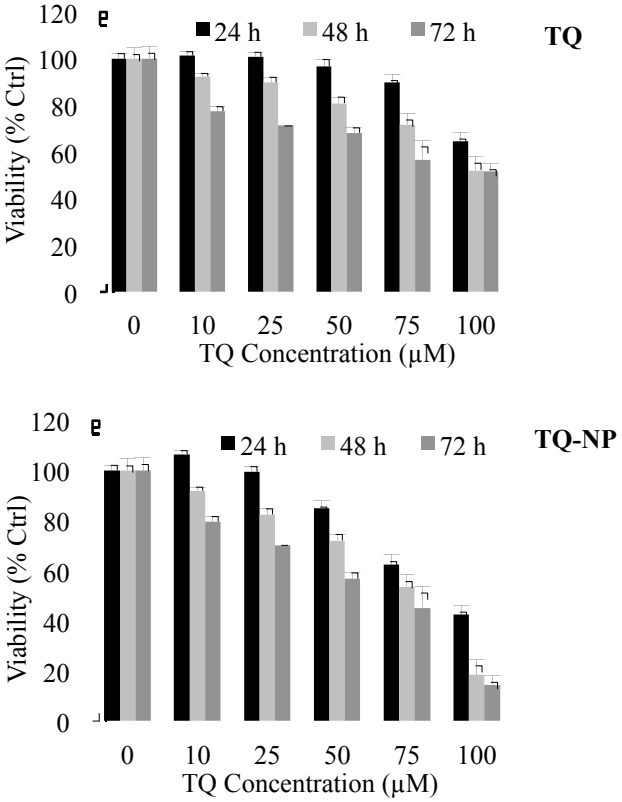

Figure 2: Effect of increasing TQ concentrations in TQ (top chart) and TQ-NP (bottom chart) formulations on the viability of MDA-MB-231 cells as determined by MTT assay at 24, 48 and 72 h post-treatment.

In the case of MDA-MB-231 cells treated with TQ (Figure 2, top), the concentration effect is noticed at TQ concentrations of 75 and 100 μM for the 24 h time point, and lower concentrations for the 48 h and 72 h time points. For the MDA-MB-231 cells treated with TQ-NP (Figure 2, bottom), the time effect is pronounced at all TQ concentrations tested (10 μM to 100 μM) for the 24 h and 48 h time points, with some variation observed between the 48 h and 72 h time points at concentrations of 50 μM and below.

TQ formulated as TQ-NP showed higher potency compared with free TQ for MCF-7 and MDA-MB-231 cell lines. The results at 72 h for TQ and TQ-NP are shown in Figure 3 for both cell lines.

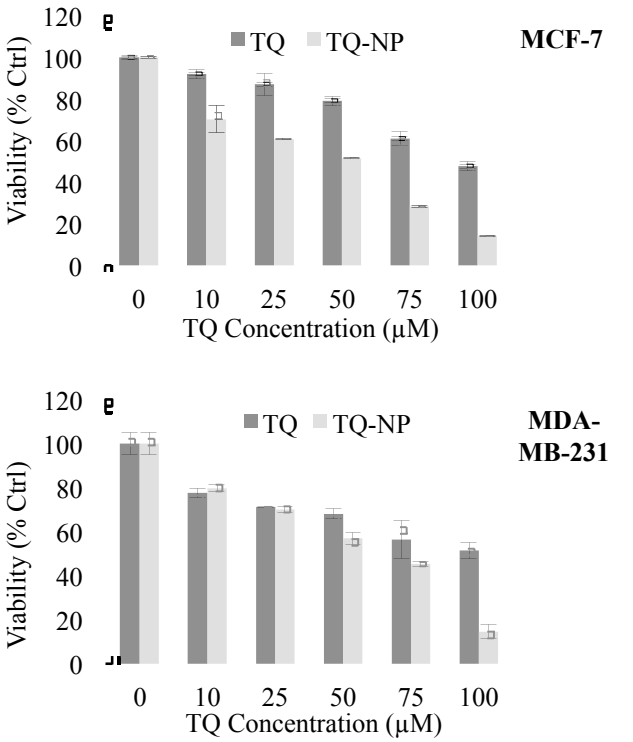

Figure 3: Effect of TQ and TQ-NP on the viability of MCF-7 (top chart) and MDA-MB-231 (bottom chart) cells as determined by MTT assay 72 hours post-treatment.

TQ-NP were more active than TQ at all time points. Specifically, treatment of MCF-7 cells with TQ concentrations in the range of 10-100 μM using TQ-NP were significantly more potent in killing cells than treatment with the same concentrations of free TQ (Figure 3, top).

Similarly, MDA-MB-231 cells were more sensitive to treatment with 50-100 μM TQ-NP than treatment with TQ (Figure 3, bottom). These results suggest that TQ-NP are a successful system for the enhancement of TQ antitumor activity *in vitro*.

4 CONCLUSION

TQ was formulated into stable nanoparticles using FNP at a TQ/polymer weight ratio of 1/1. The *in vitro* cell viability of MCF-7 and MDA-MB-231 cell lines was assessed following exposure to TQ and TQ-NP at 24, 48, and 72 h. The results reveal higher potency for TQ-NP compared with free TQ for both cell lines. The potentially added benefit of TQ-NP lies in the enhanced permeability and retention effect observed *in vivo*. The efficacy of nanoparticle based formulations of cancer drugs has already been established *in vivo* [3]. Future studies will reveal the efficacy of the TQ-NP formulation following *in vivo* administration.

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